

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :  C12Q 1/68, C12N 15/10		A1	(11) International Publication Number: WO 92/04470  (43) International Publication Date: 19 March 1992 (19.03.92)
<p>(21) International Application Number: PCT/GB91/01563  (22) International Filing Date: 12 September 1991 (12.09.91)</p> <p>(30) Priority data: 9019946.4 12 September 1990 (12.09.90) GB 9112911.4 14 June 1991 (14.06.91) GB</p> <p>(71) Applicant (<i>for all designated States except US</i>): SCIENTIFIC GENERICS LIMITED [GB/GB]; King's Court, Kirkwood Road, Cambridge CB4 2PF (GB).</p> <p>(72) Inventor; and (75) Inventor/Applicant (<i>for US only</i>): STANLEY, Christopher, John [GB/GB]; 12A Cromwell Place, St. Ives, Huntingdon, Cambridgeshire PE17 4JB (GB).</p>		<p>(74) Agent: SMART, Peter, John; W.H. Beck, Greener &amp; Co., 7 Stone Buildings, Lincoln's Inn, London WC2A 3SZ (GB).</p> <p>(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU+, TD (OAPI patent), TG (OAPI patent), US.</p>	

Published

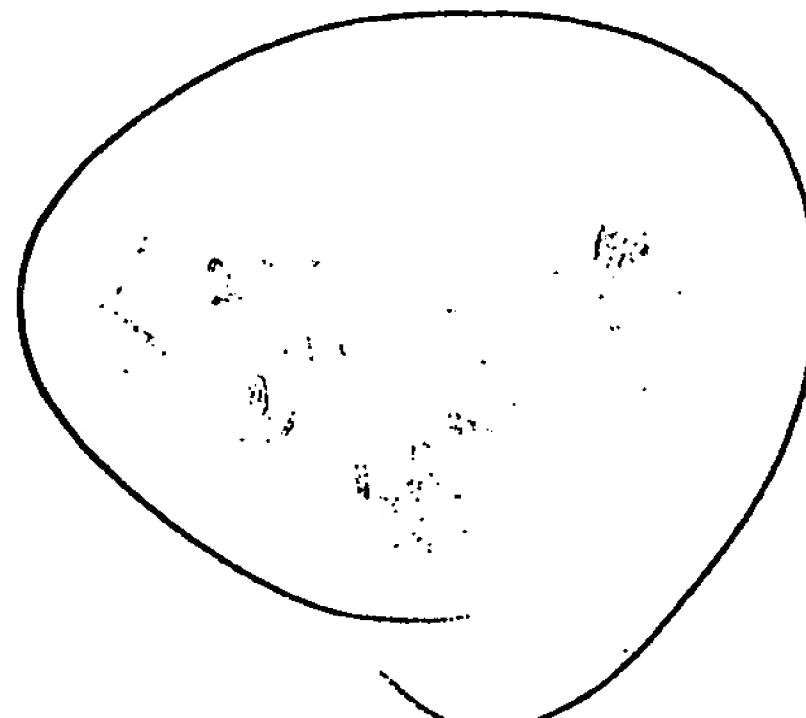
*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: ELECTROCHEMICAL DENATURATION OF DOUBLE-STRANDED NUCLEIC ACID

(57) Abstract

A process is described for denaturing native double-stranded nucleic acid material into its individual strands in an electrochemical cell. The process is an electrical treatment of the nucleic acid with a voltage applied to the nucleic acid material by an electrode. The process may also employ a promotor compound such as methyl viologen to speed denaturation. The process may be used in the detection of nucleic acid by hybridising with a labelled probe or in the amplification of DNA by a polymerase chain reaction or ligase chain reaction.



#### + DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

#### *FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	CB	United Kingdom	MW	Malawi
BG	Bulgaria	CN	Guinea	NL	Netherlands
BJ	Benin	CR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU*	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

### Electrochemical denaturation of double-stranded nucleic acid

This invention relates to processes for the treatment of nucleic acid material in order to effect a complete or partial change from double stranded form to single stranded form and to processes of amplifying or detecting nucleic acids involving such denaturation processes.

Double stranded DNA (deoxyribonucleic acid) and DNA/RNA (ribonucleic acid) and RNA/RNA complexes in the familiar double helical configuration are stable molecules that, *in vitro*, require aggressive conditions to separate the complementary strands of the nucleic acid. Known methods that are commonly employed for strand separation require the use of high temperatures of at least 60° celsius and often 100° celsius for extended periods of ten minutes or more or use an alkaline pH of 11 or higher. Other methods include the use of helicase enzymes such as Rep protein of *E.coli* that can catalyse the unwinding of the DNA in an unknown way, or binding proteins such as 32-protein of *E.coli* phage T4 that act to stabilise the single stranded form of DNA. The denatured single stranded DNA produced by the known processes of heat or alkali is used commonly for hybridisation studies or is subjected to amplification cycles.

US Patent No 4683202 (Kary B Mullis et al, assigned to Cetus Corporation) discloses a process for amplifying and detecting a target nucleic acid sequence contained in a nucleic acid or mixture thereof by separating the complementary strands of the nucleic acid, hybridising with specific oligonucleotide primers, extending the primers with a polymerase to form complementary primer extension products and then using those extension products for the further synthesis of the desired nucleic acid sequence by allowing hybridisation with the specific oligonucleotides primers to take place again. The process can be carried out repetitively to generate large quantities of the required nucleic acid sequence from even a single molecule of the starting material.

-2-

Separation of the complementary strands of the nucleic acid is achieved preferably by thermal denaturation in successive cycles, since only the thermal process offers simple reversibility of the denaturation process to reform the double stranded nucleic acid, in order to continue the amplification cycle. However the need for thermal cycling of the reaction mixture limits the speed at which the multiplication process can be carried out owing to the slowness of typical heating and cooling systems. It also requires the use of special heat resistant polymerase enzymes from thermophilic organisms for the primer extension step if the continuous addition of heat labile enzyme is to be avoided. It limits the design of new diagnostic formats that use the amplification process because heat is difficult to apply in selective regions of a diagnostic device and it also can be destructive to the structure of the DNA itself because the phosphodiester bonds may be broken at high temperatures leading to a collection of broken single strands. It is generally believed that the thermophilic polymerases in use today have a lower fidelity ie. make more errors in copying DNA than do enzymes from mesophiles. It is also the case that thermophilic enzymes such as TAQ polymerase have a lower turnover number than heat labile enzymes such as the Klenow polymerase from E.coli. In addition, the need to heat to high temperatures, usually 90° celsius or higher to denature the nucleic acid leads to complications when small volumes are used as the evaporation of the liquid is difficult to control. These limitations have so far placed some restrictions on the use of the Mullis et al process in applications requiring very low reagent volumes to provide reagent economy, in applications where the greatest accuracy of copy is required such as in the Human Genome sequencing project and in the routine diagnostics industry where reagent economy, the design of the assay format and the speed of the DNA denaturation/renaturation process are important.

-3-

Denaturation/renaturation cycles are also required in order to perform the so-called ligase chain reaction described in EP-A-0320308 in which amplification is obtained by ligation of primers hybridised to template sequences rather than by extending them.

It is known that DNA has electrochemical properties. For example, N. L. Palacek (in "Electrochemical Behaviour of Biological Macromolecules", Bioelectrochemistry and Bioenergetics, 15, (1986), 275-295) discloses the electrochemical reduction of adenine and cytosine in thermally denatured single stranded DNA at about -(minus) 1.5V on the surface of a mercury electrode. This reduction process also requires a prior protonation and therefore takes place at a pH below 7.0. The primary reduction sites of adenine and cytosine form part of the hydrogen bonds in the Watson-Crick base pairs. Palacek was unable to demonstrate reduction of adenine and cytosine in intact, native double stranded DNA at the mercury electrode. Palacek has further demonstrated that to a very limited extent the DNA double helix is opened on the surface of the mercury electrode at a narrow range of potentials centred at -(minus)1.2 V in a slow process involving an appreciable part of the DNA molecule. This change in the helical structure of the DNA is thought to be due to prolonged interaction with the electrode charged to certain potentials and is not thought to be a process involving electron transfer to the DNA. No accumulation of single stranded DNA in the working solution was obtained and no practical utility for the phenomenon was suggested. Palacek also reports that the guanine residues in DNA can be reduced at -(minus)1.8 V to dihydroguanine which can be oxidised back to guanine at around -(minus)0.3 V. The reducible guanine double bond is not part of the hydrogen bonds in the Watson-Crick base pairs and this electrochemical process involving guanine does not affect the structure of the DNA double helix.

In an earlier paper F. Jelen and E. Palacek (in "Nucleotide Sequence-Dependent Opening of Double-Stranded DNA

-4-

at an Electrically Charged Surface", Gen. Physiol. Biophys., (1985), 4, pp 219-237), describe in more detail the opening of the DNA double helix on prolonged contact of the DNA molecules with the surface of a mercury electrode. The  
5 mechanism of opening of the helix is postulated to be anchoring of the polynucleotide chain via the hydrophobic bases to the electrode surface after which the negatively charged phosphate residues of the DNA are strongly repelled from the electrode surface at an applied potential close to  
10 -(minus)1.2 V, the strand separation being brought about as a result of the electric field provided by the cathode. There is no disclosure of separating the strands of the DNA double helix while the DNA is in solution (rather than adsorbed onto the electrode) and there is no disclosure of useful amounts  
15 of single strand DNA in solution. Furthermore, there is no disclosure that the nucleotide base sequence of the DNA on the electrode is accessible from solution. The bases themselves are tightly bound to the mercury surface. A mercury electrode is a complex system and the electrode can only be operated in  
20 the research laboratory with trained technical staff.

H W Nurnberg ("Applications of Advanced Voltammetric Methods in Electrochemistry" in "Bioelectrochemistry", Plenum Inc (New York), 1983, pp. 183-225) discloses partial helix opening of adsorbed regions of native DNA to a mercury electrode surface to form a so-called ladder structure. However, the DNA is effectively inseparably bound to or adsorbed onto the electrode surface. In this condition, it is believed that the denatured DNA is of no use for any subsequent process of amplification or hybridisation analysis.  
25 To be of any use, the denatured DNA must be accessible to subsequent processes and this is conveniently achieved if the single stranded DNA is available in free solution or is associated with the electrode in some way but remains accessible to further processes. Nurnberg has not demonstrated  
30 the ability of the mercury electrode to provide useful quantities of single stranded DNA.  
35

-5-

V. Brabec and K. Niki ("Raman scattering from nucleic acids adsorbed at a silver electrode" in Biophysical Chemistry (1985), 23, pp 63-70) have provided a useful summary of the differing views from several workers on DNA denaturation at 5 the surface of both mercury and graphite electrodes charged to negative potentials. There has emerged a consensus amongst the research workers in this field that the denaturation process only takes place in DNA that is strongly adsorbed to the electrode surface and only over prolonged periods of 10 treatment with the appropriate negative voltage, a positive voltage having no effect on the double helix.

Brabec and Palacek (J. Electroanal. Chem., 88 (1978) 373-385) disclose that sonicated DNA damaged by gamma radiation is transiently partially denatured on the surface of a mercury 15 pool electrode, the process being detectable by reacting the single stranded products with formaldehyde so as to accumulate methylated DNA products in solution. Intact DNA did not show any observable denaturation.

The present invention provides a process for denaturing 20 double-stranded nucleic acid which comprises operating on solution containing nucleic acid with an electrode under conditions such as to convert a substantial portion of said nucleic acid to a wholly or partially single stranded form.

It has been found that it is possible to produce the 25 denaturation of undamaged (i.e. non-irradiated) DNA at ambient temperature by applying a suitable voltage to a solution in which the DNA is present under suitable conditions.

The mechanism for the process has not yet been fully elucidated. It is believed that the process is one in which 30 the electric field at the electrode surface produces the denaturation of the double helix when the DNA is in close proximity to the electrode but not bound irreversibly to it.

The process is found to be readily reversible. In polymerase chain reaction processes exemplified hereafter, it 35 is shown that the denatured DNA produced by the denaturing process of the invention is immediately in a suitable state

-6-

for primer hybridisation and extension. On a larger scale, it is found that samples of denatured DNA produced using a negative voltage electrode can be caused or encouraged to renature by reversal of the voltage or by incubation at a 5 higher temperature to encourage reannealing.

Preferably, according to the invention, the single stranded nucleic acid produced is free from the electrode, e.g. in solution. However, the nucleic acid may be immobilised on the electrode in double or single stranded form 10 prior to the application of the electric potential, e.g. attached by the end or a small portion intermediate the ends of the nucleic acid chain, so as to leave substantial segments of the nucleic acid molecules freely pendant from the electrode surface before and after denaturation.

15 Preferably, a potential of from -0.5 to -1.5 V is applied to said working electrode with respect to the solution, more preferably from -0.8 to -1.1 V, e.g. about -1.0 V.

Working electrode voltages are given throughout as if measured or as actually measured relative to a calomel 20 reference electrode (BDH No. 309.1030.02).

In addition to said electrode and a counter-electrode, a reference electrode may be contacted with said solution and a voltage may be applied between said electrode and said counter-electrode so as to achieve a desired controlled 25 voltage between said electrode and said reference electrode. The electrodes may be connected by a potentiostat circuit as is known in the electrochemical art.

The ionic strength of said solution is preferably no more than 250 mM, more preferably no more than 100 mM. As it has 30 been found that the rate of denaturation increases as the ionic strength is decreased, the said ionic strength is still more preferably no more than 50 mM, e.g. no more than 25 mM or even no more than 5 mM. Generally, the lower the ionic strength, the more rapid is the denaturation. However, in calculating ionic strength for these purposes it may be 35

appropriate to ignore the contribution to ionic strength of any component which acts as a promoter as described below.

The solution may contain or the electrode may have on its surface a promoter compound which assists said denaturation.

5 Although the invented process can take place in a solution containing only the electrode and the nucleic acid dissolved in water optionally containing a suitable buffer, the process can be facilitated by the presence in the solution containing the nucleic acid of such a promoter compound.

10 The compound may act as a promoter serving either to destabilise the double-stranded nucleic acid, for instance by intercalation into the double helix, or to stabilise the single-stranded form, or else to facilitate interaction between the electrode surface and the nucleic acid. By way 15 of analogy, it has been found that 4,4'-bipyridyl promotes the reduction of cytochrome C at a gold electrode even though bipyridyl is in itself electroinactive at the voltages used. (M.J. Eddowes and H.A. Hill, J. Chem. Soc. Chem. Commun. (1977), 771).

20 The promoter may be any inorganic or organic molecule which increases the rate or extent of denaturation of the DNA double helix. It should be soluble in the chosen solvent. It preferably does not affect or interfere with DNA or other materials (such as enzymes or oligonucleotide probes) which 25 may be present in the solution. Alternatively the promoter may be immobilised to or included in the material from which the electrode is constructed.

In experiments, it has been found that the promoter may 30 be a water soluble compound of the bipyridyl series, especially a viologen such as methyl viologen or a salt thereof.

35 It is believed that the positively charged viologen molecules interact between the negatively charged DNA and the negatively charged cathode to reduce electrostatic repulsion therebetween and hence to promote the approach of the DNA to the electrode surface where the electrical field is at its

-8-

strongest. Accordingly, we prefer to employ as promoters compounds having spaced positively charged centres, e.g. bipolar positively charged compounds. Preferably, the spacing between the positively charged centres is similar to that in viologens. Other suitable viologens include ethyl viologen 5 isopropyl viologen and benzyl viologen.

The process may be carried out in an electrochemical cell of the type described by C. J. Stanley, M. Cardosi and A.P.F Turner "Amperometric Enzyme Amplified Immunoassays" J. 10 Immunol. Meth (1988) 112, 153-161 in which there is a working electrode, a counter electrode and optionally a reference electrode. The working electrode at or by which the denaturing nucleic acid is effected may be of any convenient material e.g. a noble metal such as gold or platinum, or a glassy 15 carbon electrode.

The electrode may be a so called "modified electrode" in which the denaturing is promoted by a compound coated onto, or adsorbed onto, or incorporated into the structure of the electrode which is otherwise of an inert but conducting 20 material. In an alternative electrochemical cell configuration the working, counter and reference electrodes may be formed on a single surface e.g. a flat surface by any printing method such as thick film screen printing, ink jet printing, or by using a photo-resist followed by etching. It 25 is also possible that the working and reference electrodes can be combined on the flat surface leading to a two electrode configuration where the reference also acts as the counter. Alternatively the electrodes may be formed on the inside surface of a well which is adapted to hold liquid. such a well 30 could be the well known 96 well or Microtitre plate, it may also be a test tube or other vessel. Electrode arrays in Microtitre plates or other moulded or thermoformed plastic materials may be provided for multiple nucleic acid denaturation experiments.

35 The strand separation may be carried out in an aqueous medium or in a mixture of water with an organic solvent such as dimethylformamide. The use of polar solvents other than

-9-

water or non-polar solvents is also acceptable but is not preferred. The process may be carried out at ambient temperatures or if desired temperatures up to adjacent the pre-melting temperature of the nucleic acid. The process may 5 be carried out at pH's of from 3 to 10 conveniently about 7. Generally, more rapid denaturation is obtained at lower pH. For some purposes therefore a pH somewhat below neutral, e.g about pH 5.5 may be preferred. The nucleic acid may be dissolved in an aqueous solution containing a buffer whose 10 nature and ionic strength are such as not to interfere with the strand separation process.

The denaturing process according to the invention may be incorporated as a step in a number of more complex processes, e.g. procedures involving the analysis and/or the 15 amplification of nucleic acid. Some examples of such applications are described below.

The invention includes a process for detecting the presence or absence of a predetermined nucleic acid sequence in a sample which comprises: denaturing a sample double-stranded nucleic acid by means of a voltage applied to the 20 sample in a solution by means of an electrode; hybridising the denatured nucleic acid with an oligonucleotide probe for the sequence; and determining whether the said hybridisation has occurred.

Thus, the invented process has application in DNA and RNA hybridisation where a specific gene sequence is to be identified e.g. specific to a particular organism or specific to a particular hereditary disease of which sickle cell anaemia is an example. To detect a specific sequence it is 25 first necessary to prepare a sample of DNA, preferably of purified DNA, means for which are known, which is in native double stranded form. It is then necessary to convert the double stranded DNA to single stranded form before a hybridisation step with a labelled nucleotide probe which has 30 a complementary sequence to the DNA sample can take place. The denaturation process of the invention can be used for this 35

-10-

purpose in a preferred manner by carrying out the following steps:

- denaturing a sample of DNA by applying a voltage by means of an electrode to the sample DNA with optionally a promoter in solution or bound to or part of the structure of the electrode;
- hybridising the denatured DNA with a directly labelled or indirectly labelled nucleotide probe complementary to the sequence of interest; and
- determining whether the hybridisation has occurred, which determination may be by detecting the presence of the probe, the probe being directly radio-labelled, fluorescent labelled, chemiluminescent labelled or enzyme-labelled or being an indirectly labelled probe which carries biotin for example to which a labelled avidin or avidin type molecule can be bound later.

In a typical DNA probe assay it is customary to immobilise the sample DNA to a membrane surface which may be composed of neutral or charged nylon or nitrocellulose. The immobilisation is achieved by charge interactions or by baking the membrane containing DNA in an oven. The sample DNA can be heated to high temperature to ensure conversion to single stranded form before binding to the membrane or it can be treated with alkali once on the membrane to ensure conversion to the single stranded form. The disadvantages of the present methods are:

- heating to high temperatures to create single stranded DNA can cause damage to the sample DNA itself.
- the use of alkali requires an additional step of neutralisation before hybridisation with the labelled probe can take place.

One improved method for carrying out DNA probe hybridisation assays is the so called "sandwich" technique where a specific oligonucleotide is immobilised on a surface. The surface having the specific oligonucleotide thereon is

-11-

then hybridised with a solution containing the target DNA in a single-stranded form, after which a second labelled oligonucleotide is then added which also hybridises to the target DNA. The surface is then washed to remove unbound 5 labelled oligonucleotide, after which any label which has become bound to target DNA on the surface can be detected later.

This procedure can be simplified by using the denaturing process of the invention to denature the double-stranded DNA 10 into the required single-stranded DNA. The working electrode, counter electrode and optionally a reference electrode and/or a promoter can be incorporated into a test tube or a well in which the DNA probe assay is to be carried out. The DNA sample and oligonucleotide probes can then be added and the voltage 15 applied to denature the DNA. The resulting single-stranded DNA is hybridised with the specific oligonucleotide immobilised on the surface after which the remaining stages of a sandwich assay are carried out. All the above steps can take place without a need for high temperatures or addition 20 of alkali reagents as in the conventional process.

The electrochemical denaturation of DNA can be used in the amplification of nucleic acids, e.g. in a polymerase chain reaction or ligase chain reaction amplification procedure. Thus the present invention provides a process for replicating 25 a nucleic acid which comprises: separating the strands of a sample double stranded nucleic acid in solution under the influence of an electrical voltage applied to the solution from an electrode; hybridising the separated strands of the nucleic acid with at least one oligonucleotide primer that 30 hybridises with at least one of the strands of the denatured nucleic acid; synthesising an extension product of the or each primer which is sufficiently complementary to the respective strand of the nucleic acid to hybridise therewith; and separating the or each extension product from the nucleic acid 35 strand with which it is hybridised to obtain the extension product.

-12-

In such a polymerase mediated replication procedure, e.g. a polymerase chain reaction procedure, it may not be necessary in all cases to carry out denaturation to the point of producing wholly single-stranded molecules of nucleic acid.

5 It may be sufficient to produce a sufficient local and/or temporary weakening or separation of the double helix in the primer hybridisation site to allow the primer to bind to its target. Once the primer is in position on a first of the target strands, rehybridisation of the target strands in the 10 primer region will be prevented and the other target strand may be progressively displaced by extension of the primer or by further temporary weakening or separation processes.

Preferably, the said amplification process further comprises repeating the procedure defined above cyclicly, e.g. 15 for more than 10 cycles, e.g. up to 20 or 30 cycles. In the amplification process the hybridisation step is preferably carried out using two primers which are complementary to different strands of the nucleic acid.

The denaturation to obtain the extension products as well 20 as the original denaturing of the target nucleic acid is preferably carried out by applying to the solution of the nucleic acid a voltage from an electrode.

The process may be a standard or classical PCR process 25 for amplifying at least one specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids wherein each nucleic acid consists of two separate complementary strands, of equal or unequal length, which process comprises:

(a) treating the strands with two oligonucleotide primers, 30 for each different specific sequence being applied, under conditions such that for each different sequence being amplified an extension product of each primer is synthesised which is complementary to each nucleic acid strand, wherein said primers are selected so as to be substantially complementary to different strands of each specific sequence 35 such that the extension product synthesised from one primer,

-13-

when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer:

- 5 (b) separating the primer extension products from the templates on which they were synthesised to produce single-stranded molecules by applying a voltage from an electrode to the reaction mixture: and
- 10 (c) treating the single-stranded molecules generated from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesised using each of the single strands produced in step (b) as a template.

15 Alternatively, the process may be any variant of the classical or standard PCR process, e.g. the so-called "inverted" or "inverse" PCR process or the "anchored" PCR process.

20 The invention therefore includes an amplification process as described above in which a primer is hybridised to a circular nucleic acid and is extended to form a duplex which is denatured by the denaturing process of the invention, the amplification process optionally being repeated through one or more additional cycles.

25 More generally, the invention includes a process for amplifying a target sequence of nucleic acid comprising hybridisation, amplification and denaturation of nucleic acid (e.g. cycles of hybridising and denaturing) wherein said denaturation is produced by operating on a solution containing said nucleic acid with an electrode.

30 The process of the invention is applicable to the ligase chain reaction. Accordingly, the invention includes a process for amplifying a target nucleic acid comprising the steps of:  
35 (a) providing nucleic acid of a sample as single-stranded nucleic acid;  
(b) providing in the sample at least four nucleic acid probes, wherein: i) the first and second of said probes are primary probes, and the third and fourth of said probes are secondary nucleic acid probes; ii) the first probe is a single

-14-

strand capable of hybridising to a first segment of a primary strand of the target nucleic acid; iii) the second probe is a single strand capable of hybridising to a second segment of said primary strand of the target nucleic acid; iv) the 5' end 5 of the first segment of said primary strand of the target is positioned relative to the 3' end of the second segment of said primary strand of the target to enable joining of the 3' end of the first probe to the 5' end of the second probe, when said probes are hybridised to said primary strand of said 10 target nucleic acid; v) the third probe is capable of hybridising to the first probe; and iv) the fourth probe is capable of hybridising to the second probe; and  
(c) repeatedly or continuously: i) hybridising said probes 15 with nucleic acid in said sample; ii) ligating hybridised probes to form reorganised fused probe sequences; and iii) denaturing DNA in said sample by applying a voltage from an electrode to the reaction mixture.

In all of the amplification procedures described above the denaturation of the DNA to allow subsequent hybridisation 20 with the primers can be carried out by the application of an appropriate potential to the electrode. The process may be carried out stepwise involving successive cycles of denaturation or renaturation as in the existing thermal methods of PCR and LCR, but it is also possible for it to be 25 carried out continuously since the process of chain extension or ligation by the enzyme and subsequent strand separation by the electrochemical process can continue in the same reaction as nucleic acid molecules in single-stranded form will be free to hybridise with primers once they leave the denaturing 30 influence of the electrode. Thus, provided that the primer will hybridise with the DNA an extension or ligation product will be synthesised. The electrochemical DNA amplification technique can be used analytically to detect and analyse a very small sample of DNA eg a single copy gene in an animal 35 cell or a single cell of a bacterium.

-15-

The invention includes a kit for use in a process of detecting the presence or absence of a predetermined nucleic acid sequence in a sample which kit comprises, an electrode, a counter electrode and optionally a reference electrode, and 5 an oligonucleotide probe for said sequence. The probe may be labelled in any of the ways discussed above.

The invention also includes a kit for use in a process of nucleic acid amplification comprising an electrode, a counter electrode and optionally a reference electrode, and 10 at least one primer for use in a PCR procedure, or at least one primer for use in an LCR procedure, and/or a polymerase or a ligase, and/or nucleotides suitable for use in a PCR process.

15 Preferably, such kits includes a cell containing the electrodes. Preferably the kits include a suitable buffer for use in the detection or amplification procedure.

The invention will now be described with reference to the following drawings and examples.

20 Figure 1 is a diagram of an electrochemical cell used for denaturation of DNA;

Figure 2 is a drawing of an electrophoresis gel showing the movement of single and double stranded DNA;

25 Figure 3 is a diagram of a electrophoresis gel showing the electrical denaturation of calf thymus DNA in the absence of any promoter such as methyl viologen;

Figure 4 is a diagram of a electrophoresis gel showing the renaturation of denatured calf thymus DNA;

30 Figure 5 is a drawing of an electrophoresis gel showing the time course of the thermal denaturation of linear double stranded DNA from the bacteriophage M13;

Figure 6 is a drawing of an electrophoresis gel showing the time course of the electrical denaturation of linear M13 DNA;

35 Figure 7 is a drawing of an electrophoresis gel showing a comparison of a thermally amplified segment of M13 with an

-16-

equivalent electrically amplified segment of M13 in the presence of methyl viologen;

5 Figure 8 is a drawing of an electrophoresis gel showing a comparison of a thermally amplified segment of M13 with an equivalent electrically amplified segment in the absence of methyl viologen.

10 Figure 9 is a drawing of an electrophoresis gel showing a fragment of 'bluescript' DNA amplified using electrical PCR in the presence of methyl viologen; and

15 Figure 10 is a drawing of an electrophoresis gel showing amplified fragments of two 'bluescript' DNA's produced by electrical PCR in the absence of methyl viologen.

20 In Figure 1 there is shown a cell structure 10 comprising a working compartment 12 in which there is a body of DNA-containing solution, a working electrode 14, a counter electrode 16, a FiVac seal 19, a Kwik fit adaptor 21 and a magnetic stirrer 18. A reference electrode 20 in a separate side arm is connected via a "luggin" capillary 23 to the solution in the sample 12. The working electrode, counter electrode and reference electrode are connected together in a potentiostat arrangement so that a constant voltage is maintained between the working electrode 14 and the reference electrode 20. Such potentiostat arrangements are well known (see for example "Instrumental Methods in Electrochemistry" 25 by the Southampton Electrochemistry Group, 1985, John Wiley and Sons, p 19).

30 The electrode 14 is a circular glassy carbon rod of diameter 0.5 cm, narrowing to 0.25 cm at a height of 10mm, and having an overall length of 9 cm inside a teflon sleeve of outside diameter 0.8cm (supplied by Oxford Electrodes, 18 Alexander Place, Abingdon, Oxon), and the reference electrode 16 is a 2 mm pin calomel (supplied by BDH No 309/1030/02). The counter electrode is supported by a wire which is soldered to a brass sleeve 25 above the adaptor and passes down and exits the teflon sleeve 20 mm from the base of the working electrode. The wire attaches to a cylindrical platinum mesh

-17-

counter-electrode supplied by Oxford Electrodes which annularly surrounds the working electrode.

This cell is used in the following examples.

EXAMPLE 1

5        In this example of DNA denaturation, the two methods of thermal and electrical denaturation have been compared. To achieve electrical denaturation, 1.60 ml of a solution of methyl viologen dichloride at 1mg/ml in distilled water (adjusted to pH 7 by titration with 0.1 M sodium hydroxide) 10 was added to the working compartment of the electrochemical cell described above. The reference arm of the cell in which the reference electrode 20 resides contained 0.4 ml of this solution. A sample of 120 µl of a stock solution of calf 15 thymus DNA (Sigma Chemical Company catalogue number: D4522, average fragment size 5,000 bases) at 1 mg/ml in distilled water (the pH was not adjusted) was added to the working chamber of the electrochemical cell to give a 70 µg/ml final DNA concentration. The total ionic strength of the solution was calculated to be approximately 5 mM. A voltage of 20 -(minus)1.0 V was applied between the working electrode and the reference electrode.

The electrochemical cell was left for 16 hours at room temperature (22°C) with continuous gentle stirring. On applying the potential to the working electrode 14 the blue colour of reduced methyl viologen was observed in the immediate vicinity of the working electrode. A 100 µl sample from the working compartment of the electrochemical cell was taken at the end of the experiment and prepared for gel electrophoresis analysis by mixing with 20 µl of gel loading 25 buffer which was the same as is described below for the gel itself which also contained 0.25 % (w/v) bromophenol blue (BDH Indicators 20170), 0.25 % (w/v) xylene cyanol (Sigma X2751), and 30 % (v/v) glycerol (BDH AnalaR 100118). The mixtures were stored on ice prior to loading 10 µl samples in the wells 30 cast in an agarose electrophoresis gel.

For the thermal denaturation experiment the DNA solution

-18-

at 1 mg/ml in distilled water (the pH was not adjusted) was heated to 100°C for 10 minutes in a boiling water bath. The tube containing the thermally denatured DNA was then removed from the water bath and placed immediately into a beaker 5 containing an ice/water mix to ensure rapid cooling to prevent renaturation of the sample back to the double stranded form. A 100 µl sample of the thermally denatured DNA solution was prepared for gel electrophoresis by mixture with 20 µl of gel loading buffer which contained 0.25% (w/v) bromophenol blue, 10 0.25 % (w/v) xylene cyanol and 30 % (w/v) glycerol.

Native intact calf thymus DNA was also prepared for gel electrophoresis by mixing 100 µl of the starting solution of DNA (before thermal or denaturation) with 20 µl of gel loading buffer and stored on ice until required.

15 The gel (a section of which is shown in Figure 2) had a number of wells 30 into which the samples could be loaded, and 10 µl samples were placed into individual wells. The gel had a total volume of 50 ml and was 10 cm wide and 75 cm long; it was 0.5 % (w/v) agarose in 0.089 M tris buffer pH 8.0 20 containing 0.1 M borate and 0.01 M sodium EDTA. The gel was run for 85 minutes at an applied constant voltage of 55 volts using a power supply from Pharmacia No 500/400. The gel was then removed from the electrophoresis apparatus and stained by addition of 0.75 ml of ethidium bromide (Pharmacia No 1840- 25 501, lot 9503860E) at 20 µg/ml in distilled water. After staining for 30 minutes the gel was washed in distilled water.

The stained gel was trans-illuminated with ultraviolet light and then photographed with a Polaroid instant camera system using a red filter to reduce background from the UV source.

30 The gel shown in Figure 1 has samples A, B and C. Sample A was the starting material used in the test (calf thymus DNA). Sample B was a sample of calf thymus DNA which had been electrically denatured according to the invention and sample C was a sample of thermally denatured DNA. The DNA stain ethidium bromide becomes fluorescent when it 35 intercalates into the double helical structure of intact

-19-

native DNA. Hence it can be used to identify the double stranded DNA in Figure 1. As the DNA is denatured it becomes progressively single stranded and the efficiency of staining with ethidium bromide decreases. However, there is still some residual staining of single stranded calf thymus DNA, probably because there is still some ordering of the bases or even some regions which remain double stranded to which ethidium bromide can bind. Therefore the stain is still useful in detecting denatured DNA as well as intact DNA. In Figure 1 it will be noted that the samples B and C are of much higher mobility than sample A through the gel indicating that the DNA after thermal denaturation and after electrical reduction has similar physical characteristics, showing a denaturation to the single stranded form which runs faster in this gel system. Similar results have been obtained using gold and platinum working electrodes.

#### EXAMPLE 2

Example 1 was repeated as before, but DNA samples were taken after 15 minutes, 3 hours and 22 hours treatment in the electrochemical cell in order to provide a time course of the denaturation of the DNA. During sampling from the cell the potentiostat was switched to a dummy cell represented by a resistor. A gradual progressive denaturation of the DNA into the faster migrating form on the gel was observed. The gel pattern after 15 minutes (not shown) is interpreted to represent a mixture of partially and fully denatured DNA but no evidence of wholly native DNA was seen, and in later samples the proportion of fully denatured DNA continued to increase.

#### EXAMPLE 3

In a number of runs at varying motor concentrations and at a fixed DNA concentration of 20 µg/ml in the electrochemical cell and at an ionic strength of 15 mM to 150 mM the rate of DNA denaturation was found to be greatly accelerated by increasing the promoter concentration from 3 to 30 mg/ml. At the higher concentration denaturation of the

-20-

DNA was almost complete in 15 minutes only. In this example, the overwhelmingly predominant source of ionic strength is the promotor itself.

EXAMPLE 4

5 At a fixed promoter concentration of 3 mg/ml and at an ionic strength of 15 mM (derived essentially from the promoter) the rate of denaturation at various DNA concentrations was assessed. The results show that at 4 µg/ml DNA the denaturation time was reduced to under 0.5 hours (as 10 assessed by the disappearance of the double stranded DNA band on the electrophoresis gel).

The foregoing Examples demonstrate that the ratio of DNA to the promoter methyl viologen affects the rapidity and completeness of the denaturation of the DNA. These Examples 15 were carried out at low ionic strength in distilled water. Repeating these Examples at higher ionic strength, i.e. 10 to 250 mM NaCl, lengthened the denaturation time of the DNA in solution by electrical means. This is attributed to the additional stabilisation that salt provides to the double 20 helical structure of DNA.

The foregoing Examples have also been repeated following exactly the procedure described above for calf thymus DNA with other whole genomic DNA samples from salmon testes DNA (Sigma No D1626) and with human placental DNA (Sigma No D7011). 25 Exactly the same results were obtained on the agarose electrophoresis gel with these alternative DNA sources.

EXAMPLE 5

Figure 3 shows the results of an electrical DNA denaturation carried out according to the methods described 30 above but without the inclusion of the methyl viologen promoter. The ionic strength was less than 1 mM and the calf thymus DNA concentration was 70 µg/ml. The electrochemical cell was left for a total of 20 hours at ambient temperature (22°C) with continuous gentle stirring at a potential of - 35 (minus) 1.0 V on the working electrode. Samples were taken from the cell at 1 hour, 3 hours and 4 hours as well as at the

-21-

end of the procedure. During the sampling of the DNA solution from the cell the potentiostat was switched to a dummy cell represented by a resistor in order to avoid current surges. The gel in Figure 3 shows that denaturation of the calf thymus 5 DNA to the single stranded form does indeed occur in the absence of promoter, but the rate of the process was much slower than that observed with methyl viologen present. Track A is the starting material, Track B is after 1 hour, Track C after 3 hours, Track D after 4 hours and Track E after a total 10 of 20 hours at -(minus)1 V. Some limited denaturation of the DNA was observed within 1 hour but considerable amounts of intact double stranded DNA were still visible after 4 hours and only disappeared after overnight incubation.

EXAMPLE 6

15       Figure 4 shows the results from a renaturation procedure in which electrically denatured DNA has been treated under the appropriate thermal and electrical condition to recover the original double stranded DNA. To an electrochemical cell of the type shown in Figure 1, 2.45 ml of distilled water was added to the working compartment and 0.2 ml c<sup>-</sup> distilled water was added to the reference compartment. To the working compartment were added 250 µl of calf thymus DNA at 1 mg/ml in distilled water (pH not adjusted) and 100 µl of a solution of methyl viologen at 100 mg/ml in distilled water (pH 20 adjusted to pH 7 with 0.1 M sodium hydroxide). The final DNA concentration in the solution was 90 µg/ml and the promoter 25 3.5 mg/ml. The ionic strength was approximately 15 mM derived essentially from the promoter. After gentle stirring of the contents of the cell with the magnetic stirring bar, a 100 µl sample was taken and stored on ice or frozen at -(minus) 20 °C.

30       The electrodes were positioned in the cell, as illustrated in Figure 1, and a voltage of -(minus)1 V was applied to the working electrode. The contents of the cell were stirred gently with the magnetic stirring bar and the conversion of the DNA to the single stranded form continued 35 for 90 minutes at ambient temperature (22 °C). After 90

-22-

minutes the cell was switched to dummy and two 100 µl samples were taken, one sample was stored on ice the second was incubated at 55 °C in a water bath after addition of 2 µl of 10 times concentrated "reannealing buffer" (final concentration 20 mM NaCl, 2 mM Tris HCl pH 8.7, 0.2 mM EDTA) for 25 hours. The voltage at the working electrode was reversed to +(plus)1 V and treatment of the DNA solution in the electrochemical cell proceeded for a further 25 hours. After this second time period 100 µl of the DNA solution was removed from the cell and stored on ice. Each of the 4 100 µl samples was mixed with 20 µl of gel loading buffer described above and stored on ice until required for electrophoresis.

Figure 4 is an agarose gel run exactly as described above showing the four DNA samples; A is the starting intact calf thymus material, B is the electrically denatured material, C is the electrically denatured and subsequently electrically renatured material, D is the electrically denatured material which was subsequently thermally renatured. It can be seen from the gel that both the electrically denatured thermally renatured and electrically denatured electrically renatured DNA returns to the original mobility of the double stranded starting material.

EXAMPLE 7

This example illustrates that a bacteriophage genome can be electrically denatured to a single stranded form in a manner analogous to the thermal method. Bacteriophage M13 (M13mp18RF1 Double stranded form supplied by CP Laboratories, PO Box 22, Bishop's Stortford, Herts, UK) was employed. M13 is in circular form which can adopt a number of different coiled and supercoiled configurations. This leads to a complex set of bands on the agarose electrophoresis gel. Therefore the gel pattern was simplified to a single band by subjecting M13 to a restriction digest with the enzyme BgI, which has only one restriction site on the M13 genome. To one vial of M13 as supplied by CP Laboratories (containing 10 µg of M13 in 100 µl of buffer 10 mM Tris HCl pH 7.5 1 mM EDTA)

-23-

10 µl of 'restriction buffer' was added (0.1 M Tris HCl pH 7.9, 0.01 M Magnesium Chloride, 0.05 M Sodium Chloride) and 6 µl of restriction enzyme (a stock at 8,000 U/ml supplied by CP Laboratories No 143S). The solution was incubated at 37°C  
5 in a water bath for 16 hours. To ensure the linearisation was complete 1 µl of the treated DNA was mixed with 3 µl of distilled water and 1 µl of gel loading buffer (described earlier) was run on a 1% agarose gel. If more than one band was seen after electrophoresis at 100 mA for 1 hour and  
10 subsequent staining, the DNA was redigested. Once linearity was determined the M13 was precipitated by adding 125 µl of 1 M magnesium chloride, 25 µl of 3 M sodium acetate and 125 µl of absolute ethanol (AnalaR grade from BDH Ltd, Poole, UK). The mixture was frozen in dry ice for 20 minutes for 16 hours  
15 and the precipitate was collected by 15 minute centrifugation after thawing. The pellet was washed with 0.15 ml of 70% (v/v) ethanol and collected again by centrifugation. Finally the pellet was dried under vacuum for 15 minutes and resuspended in 100 µl of distilled water.

20 The linearised M13 DNA was then subjected to both thermal and electrical denaturation. A series of tubes was set up. The series of 6 tubes contained 1 µl M13 DNA with 4 µl of distilled water. One tube was placed on ice, and the other tubes were heated for 2,4,6,8,10 minutes in a boiling water  
25 bath. After a brief period of centrifugation the tubes were placed on ice (to prevent renaturation of the thermally denatured DNA). To each tube 1 µl of gel loading buffer was added. Before being loaded and subsequently run on a 1% agarose gel at 100 mA for 1 hour each tube was briefly vortexed.  
30

Figure 5 shows the results from this thermal denaturation. Track A is the starting material (0 minutes at 100°C), Track B is 2 minutes at 100°C, Track C is 6 minutes at 100°C.

35 It can be clearly seen from the gel that the intact double stranded M13 rapidly disappears (within 4 minutes) from the gel as it denatures to single stranded form which does not

-24-

bind ethidium bromide stain with high efficiency. A faint smear remains on the gel with a higher mobility than native double stranded DNA and this may be the very faintly stained single stranded material.

5      For the electrical denaturation experiment. 920 µl of distilled water was added to an electrochemical cell as illustrated in Figure 1. 30 µl of a 100 mg/ml solution of methyl viologen in distilled water was added along with 50 µl of linearised M13 to the electrochemical cell. The solution  
10     was then mixed gently using the stirring bar. A potential of -(minus) 1.0 V was applied to the working electrode. Samples (50 µl) were taken at timed intervals, and stored on ice until required. The DNA was precipitated by adding 5 µl of 1 M Magnesium Chloride, 25 µl of 3 M Ammonium Acetate and 125 µl  
15     of absolute (100%) Ethanol to each sample. The precipitate was collected by freezing on dry ice and centrifuging as described above. The pellet was resuspended in 10 µl of distilled water and subjected to gel electrophoresis.

20     Figure 6 shows the time course of the electrical denaturation of M13. Track A is the starting material, Track B is after 5 minutes, Track C is after 15 minutes. The gel shows the loss of the double stranded DNA structure which has disappeared by 15 minutes and the appearance of the faintly stained single stranded smear.  
25     EXAMPLE 8

Figure 7 shows the results from an electrochemical cell polymerase chain reaction (PCR) carried out in the presence of the promoter methyl viologen.

30     30 µl of a methyl viologen stock at 100 mg/ml (so the working concentration of methyl viologen was 3 mg/ml) and 50 µl of stock linear M13 were added to 920 µl distilled water (pH not adjusted) in the working compartment of the cell and gentle stirring achieved by a stirring bar. 200 µl of a 3 mg/ml solution of methyl viologen was added to the reference  
35     electrode compartment of the cell. All parts of this procedure were carried out at ambient temperature (22°C). For

-25-

the initial cycle of the polymerase chain reaction a voltage of -(minus) 1 V was applied for 7 minutes and it was observed that the reduced form of the promoter methyl viologen was produced and accumulated such that all the liquid became blue.

5 Then the potentiostat was switched to dummy. The working/counter electrode was removed from the cell and the solution left stirring for 3 minutes and it was observed that the blue colour rapidly disappeared during this period.

Reagents were then added to the cell, with the stirrer bar still stirring, namely 6 $\mu$ l of primer (M13 Sequencing primer (-47)24 mer 5'(CGCCAGGGTTTCCCAGTCACGAC)3' supplied by ALTA Biosciences, University of Birmingham, UK as a 5  $\mu$ g lyophilised powder) at a final concentration of 78 pmol, 6 $\mu$ l of reverse primer M13 Reverse Sequencing primer (-24)16mer 15 5'd(AACAGTCATGACCATTG)3' supplied as a 5  $\mu$ g lyophilised powder) at a final concentration of 78 pmol, 13  $\mu$ l of decynucleotide triphosphate mix (each dNTP present at a final concentration of 26 $\mu$ M, supplied by Pharmacia Ltd., Midsummer Boulevard, Milton Keynes, UK), 4  $\mu$ l, buffer mix (at final concentration of 6.6 mM Tris HCl pH8, 1 mM MgCl<sub>2</sub>) and then 10  $\mu$ l of Klenow DNA polymerase (supplied by CP Laboratories, as 5,000 U/ml, and Northumbrian Biologicals Ltd, Cramlington Northumberland, as 5,000 U/ml). Excluding the promotor the 20 ionic strength was therefore about 20 mM. There was then a 7 minute incubation, with gentle stirring for the first 1 minute and no stirring for the next 6 minutes.

Then the working/counter electrode was replaced and the second cycle of the polymerase chain reaction started by -(minus) 1 V being applied for 5 minutes and it was observed 30 that the reduced form of the promoter methyl viologen was produced and accumulated such that all the liquid became blue. Then the potentiostat was switched to dummy. The working/counter electrode was removed from the cell and the solution left stirring for 3 minutes and it was observed that 35 the blue colour rapidly disappeared during this period. Reagents were then added to the cell, with the stirrer bar

-26-

still stirring, i.e. 13  $\mu$ l of deoxynucleotide triphosphate mix (details as above) and then 2.5  $\mu$ l of Klenow DNA polymerase (details as above). There was then a 7 minute incubation, with gentle stirring for the first 1 minute and no stirring for the next 6 minutes.

The second cycle was repeated as third to tenth cycles, omitting the adding of reagents at the end of the tenth cycle.

A sample was taken from the working compartment of the cell (750  $\mu$ l) and it was split into sub-samples for ease of processing (3  $\times$  250  $\mu$ l), then to each tube was added 50  $\mu$ l 1 M Magnesium Chloride, 98  $\mu$ l 3 M Sodium Acetate and 500  $\mu$ l 100% Ethanol. The samples were frozen on dry ice for 20 minutes and then after thawing centrifuged for 15 minutes to obtain a pellet. The pellet was washed with 250  $\mu$ l of 70% ethanol and centrifuged as described before. The pellet was dried under vacuum for 15 minutes and then the pellet in each tube was resuspended in 7  $\mu$ l distilled water (pH not adjusted) and after extensive vortexing and leaving on ice, the contents of the 3 tubes pooled before running on a gel. 4  $\mu$ l of gel loading buffer was added to the sample prior to running on a 12% polyacrylamide electrophoresis gel (made according to the following recipe for three mini gels: 42 ml distilled H<sub>2</sub>O, 8 ml TBE buffer (a stock of 500 ml distilled water containing 54 g Trizma Base (Sigma T1503) 25 g Boric acid (Sigma B0252) 20 ml 0.5 M EDTA pH8 (BDH 10093)), 24 ml 40% Acrylamide solution (BDH 44354), 6 ml of 2% N'N' methylene bis acrylamide solution (BDH 44355), 400  $\mu$ l 15% Ammonium persulphate solution in distilled water (Sigma A7262), 100  $\mu$ l TEMED (BDH 44308)).

In Figure 7, lane A contains primers, these run faster on the gel than the thermally amplified 119 base pair fragment in lane B. Lanes C and D contain 5  $\mu$ l and 15  $\mu$ l, respectively, of electrically amplified product. In lanes C and D high molecular weight M13 DNA is contained in the well, there is some smearing of DNA in the upper part of the gel, this is more pronounced in lane D. In both lanes, primers can be seen at the same mobility as in lane A, however in lane D

-27-

extensive "flaring" of the primers is observed. The amplified product can be seen in both lanes C and D at the same mobility as the thermally amplified sample in lane B.

EXAMPLE 9

5       Figure 8 shows the results from an electrochemical cell polymerase chain reaction (PCR) experiment carried out in the absence of promoter. The method used was essentially the same as for Example 8 with promoter described above except that no promoter is added to the cell and all additions of primer and  
10 reverse primer were of 3  $\mu$ l each (not 6  $\mu$ l as described above) and the experiment ran for 15 cycles.

In Figure 8 lane A contains a thermally amplified 119bp product, lane B primers, and lane E stock M13 that is confined to the well due to its high molecular weight. Lanes C and D  
15 contain the product of electrical amplification. The 119 base pair amplified region is clearly visible at the same mobility on the gel as the thermally amplified product. The primers in lanes C and D run at the same mobility as lane B. The reduction of the amount of primers added in this experiment  
20 in comparison to the experiment illustrated in Figure 7 reduces the flaring effect in the gel.

EXAMPLE 10

SK 'Blue script' (Stratagene) is a circular 2,964 base pair vector. It contains a polylinker region which contains  
25 the M13 primer binding sites between which the target region of DNA amplified in Examples 8 and 9 is located.

Before use in this Example the blue script was linearised using restriction enzyme Xmn 1; this cuts at site 2645. Thus to 80  $\mu$ moles of blue script, approximately 50 ug of DNA, 2.5  
30  $\mu$ l of -0.13 M -Tris buffer pH 7.9 containing 0.13 M magnesium chloride and 0.3 M sodium chloride and 8  $\mu$ l of Xmn 1 restriction enzyme (Stratagene) was added. The mixture was incubated at 37°C overnight. After phenol chloroform extraction and ethanol precipitation the sample was resuspended to 50  $\mu$ l in distilled water.

-28-

To the working compartment of the cell of Figure 1 was added 1.2 ml of distilled water, and 30 $\mu$ l of 100 mg/ml stock methyl viologen. 200 $\mu$ l of this mixture was pipetted into the reference compartment. 5 50  $\mu$ l (approximately 45  $\mu$ g) of restriction digested SK blue script was added to the working compartment of the cell and the electrodes were placed in their respective compartments. Denaturing was conducted for 7 minutes at -1V, and the working and counter-electrodes were removed from the cell. After 3 minutes stirring, to allow the 10 reoxidation of the methyl viologen (a lagphase), the reagents listed below were added. The nucleic acid was allowed to anneal and extend for 5 minutes, the first minute with stirring the subsequent 4 minutes in the absence of stirring. The denaturing, lag phase and reagent addition, annealing and 15 extending step were repeated for 10 cycles, but in cycles 2-10 the denaturation step was for 5 minutes.

Addition of reagents:

Cycle 1: 4  $\mu$ l of 1.65 M Tris buffer pH 8.7 containing 330 mM magnesium chloride

20

13  $\mu$ l of dATP, dGTP, dTTP and dCTP at 10 mM each in distilled water (CHASE)

10  $\mu$ l of Klenow DNA polymerase - as in Example B

25

3  $\mu$ l of each of concentrated primer (2)

Cycle 2-4 and 6-10

5  $\mu$ l of CHASE

2.5  $\mu$ l of Klenow DNA polymerase

After the 10 cycles were completed, the sample was 30 divided into three and ethanol precipitated, dried and resuspended in 15  $\mu$ l of distilled water. The sample was run on a 12% polyacrylamide gel at a constant current of 100 mA for 1 hour. The gel was stained with ethidium bromide. The gel is shown in Figure 9.

35 Both the thermally amplified product of M13, a 119 bp band, (run as a standard) and the primers can be clearly seen

-29-

in lanes B and C respectively. Due to the high concentration of DNA loaded on to lane A, the lane had high background but a band of greater than 119 bp could be clearly seen. This band is the 200 bp amplified region of SK blue script.

5    EXAMPLE 11

The procedure of Example 10 was repeated except that further restriction digests were performed to produce 20 µg of linear 3000 base pair bluescript DNA using Xmn 1, and 15 µg of 450 base pair blue script DNA using Pvu 2 (Stratagene).

10    The electrical PCR amplification process was performed in the absence of methyl viologen. A lag phase was not necessary and was omitted.

The polyacrylamide gel is shown in Figure 10.

15    Lane A contains a 'ladder marker', a set of known DNA sizes, which is used to gauge the molecular weight of experimental samples.

Lane B contains the 450 base pair electrically amplified 'blue script' DNA. The template 450 base pair band can be clearly seen, as can an amplified band of 200 base pairs.

20    Lane C contains the 3000 base pair linear electrically amplified 'blue script' DNA. The template DNA is confined to the well (as might be expected due to its 3000 base pair size) and an amplified band of 200 base pairs can clearly be seen.

25    Many modifications and variations of the invention as specifically described above are possible within the scope of the invention.

-30-

CLAIMS

1. A process for denaturing double-stranded nucleic acid which comprises operating on solution containing said nucleic acid with an electrode under condition such as to convert a substantial proportion of said nucleic acid to a wholly or partially single stranded form.
2. A process as claimed in Claim 1, wherein a potential of from -0.5 to -1.5 V is applied to said electrode with respect to said solution.
3. A process as claimed in Claim 2, wherein said voltage is from -0.8 to -1.1 V.
4. A process as claimed in any preceding claim, wherein said electrode, a reference electrode and a counter-electrode are contacted with said solution and a voltage is applied between said electrode and said counter-electrode so as to achieve a desired controlled voltage between said electrode and said reference electrode.
5. A process as claimed in any preceding claim, wherein the ionic strength of said solution is no more than 250 mM.
6. A process as claimed in Claim 5, wherein the said ionic strength is no more than 100 mM.
7. A process as claimed in Claim 5, wherein the said ionic strength is no more than 50 mM.
8. A process as claimed in Claim 5, wherein the said ionic strength is no more than 25 mM.
9. A process as claimed in Claim 5, wherein the said ionic strength is no more than 5 mM.
10. A process as claimed in any preceding claim, wherein the solution contains or the electrode has on its surface a promoter compound which assists said denaturation.
11. A process as claimed in Claim 10, wherein said compound is methyl viologen or a salt thereof.
12. A process as claimed in any preceding claim, wherein the electrode is of carbon, gold or platinum.

-31-

13. A process as claimed in any preceding claim, carried out at a temperature less than the melting point of the double-stranded nucleic acid.
14. A process as claimed in Claim 13, carried out at about 5 ambient temperatures.
15. A process as claimed in any preceding claim carried out at a pH of from 3 to 10.
16. A process as claimed in Claim 15, carried out at a pH of about 7.
- 10 17. A process as claimed in any preceding claim, wherein the nucleic acid is dissolved in an aqueous solution containing a buffer whose nature and ionic strength are such as not to interfere with strand separation of the nucleic acid.
18. A process as claimed in any preceding claim, wherein said 15 nucleic acid is DNA.
19. A process as claimed in any preceding claim, wherein said process is carried out as a denaturing step in a nucleic acid amplification procedure.
20. A process for amplifying a target sequence of nucleic acid comprising hybridisation amplification and denaturation of nucleic acid, wherein said denaturation is produced by operating on a solution containing said nucleic acid with an electrode.
21. A process as claimed in Claim 20, which is a polymerase 25 chain reaction amplification process or a ligase chain reaction amplification process.
22. A process for replicating a nucleic acid which comprises: separating the strands of a sample double stranded nucleic acid in solution under the influence of an electrical voltage applied to the solution from an electrode; hybridising the separated strands of the nucleic acid with at least one oligonucleotide primer that hybridises with at least one of the strands of the denatured nucleic acid; synthesising an extension product of the or each primer which is sufficiently 30 complementary to the respective strand of the nucleic acid to hybridise therewith; and separating the or each extension
- 35

product from the nucleic acid strand with which it is hybridised to obtain the extension product.

23. A process as claimed in Claim 22, which further involves repeating the procedure defined in Claim 22 cyclicly.

5 24. A process as claimed in Claim 22 or Claim 23, wherein the hybridisation step is carried out using two primers which are complementary to different strands of the nucleic acid.

10 25. A process as claimed in any one of Claims 22 to 24, wherein the denaturation to obtain the extension products is carried out by applying to a solution of the extension product a voltage from an electrode.

15 26. A process as claimed in Claim 22, for amplifying at least one specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids wherein each nucleic acid consists of two separate complementary strands, of equal or unequal length, which process comprises:

20 (a) treating the strands with two oligonucleotide primers, for each different specific sequence being applied, under conditions such that for each different sequence being amplified an extension product of each primer is synthesised which is complementary to each nucleic acid strand, wherein said primers are selected so as to be substantially complementary to different strands of each specific sequence such that the extension product synthesised from one primer, 25 when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer:

30 (b) separating the primer extension products from the templates on which they were synthesised to produce single-stranded molecules by applying a voltage from an electrode to the reaction mixture: and

35 (c) treating the single-stranded molecules generated from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesised using each of the single strands produced in step (b) as a template.

-33-

27. A process for amplifying a target sequence of nucleic acid comprising cycles of hybridisation and denaturation of nucleic acid wherein said denaturation is produced by operating on a solution containing said nucleic acid with an electrode.
- 5
28. A process as claimed in Claim 21 for amplifying a target nucleic acid comprising the steps of:
- (a) providing nucleic acid of a sample as single-stranded nucleic acid;
- 10
- (b) providing in the sample at least four nucleic acid probes, wherein: i) the first and second of : id probes are primary probes, and the third and fourth of said probes are secondary nucleic acid probes; ii) the first probe is a single strand capable of hybridising to a first segment of a primary strand of the target nucleic acid; iii) the second probe is a single strand capable of hybridising to a second segment of said primary strand of the target nucleic acid; iv) the 5' end of the first segment of said primary strand of the target is positioned relative to the 3' end of the second segment of
- 15
- said primary strand of the target to enable joining of the 3' end of the first probe to the 5' end of the second probe, when said probes are hybridised to said primary strand of said target nucleic acid: v) the third probe is capable of hybridising to the first probe; and iv) the fourth probe is capable of hybridising to the second probe; and
- 20
- (c) repeatedly or continuously: i) hybridising said probes with nucleic acid in said sample; ii) ligating hybridised probes to form reorganised fused probe sequences; and iii) denaturing DNA in said sample by applying a voltage from an electrode to the reaction mixture.
- 25
29. A process for detecting the presence or absence of a predetermined nucleic acid sequence in a sample which comprises: denaturing a sample double-stranded nucleic acid by means of a voltage applied to the sample in a solution by means of an electrode; hybridising the denatured nucleic acid
- 30
- 35

-34-

with an oligonucleotide probe for the sequence; and determining whether the said hybridisation has occurred.

30. A kit for use in a process of detecting the presence or absence of a predetermined nucleic acid sequence in a sample which kit comprises, an electrode, a counter electrode and optionally a reference electrode, and an oligonucleotide probe for said sequence.

10 31. A kit for use in a process of nucleic acid amplification comprising an electrode, a counter electrode and optionally a reference electrode, and at least one primer for use in a PCR procedure, or at least one primer for use in an LCR procedure, and/or a polymerase or a ligase and/or nucleotides suitable for use in a PCR process.

15

20

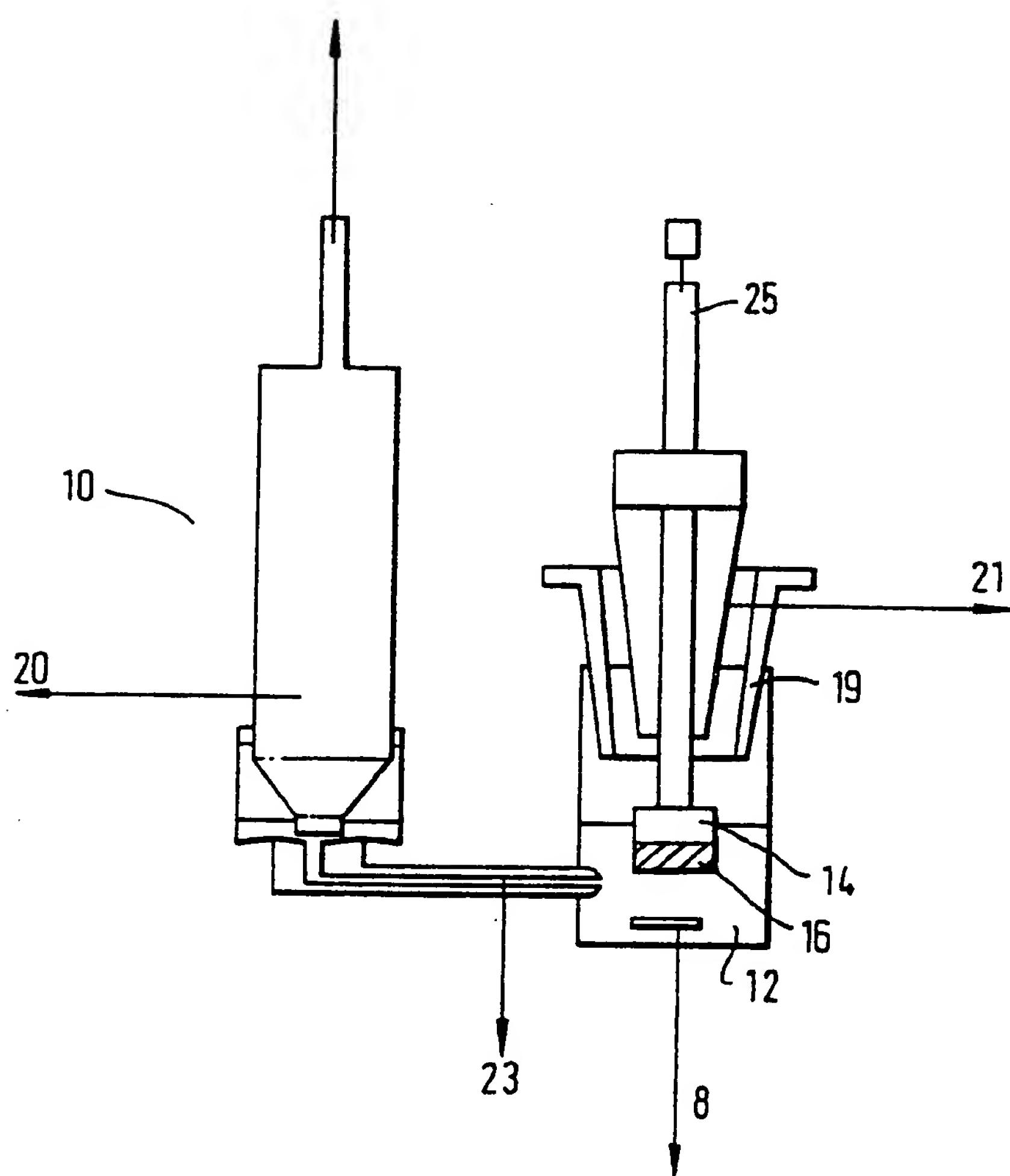
25

30

35

1 / 6

FIG. 1



2 / 6

FIG. 3

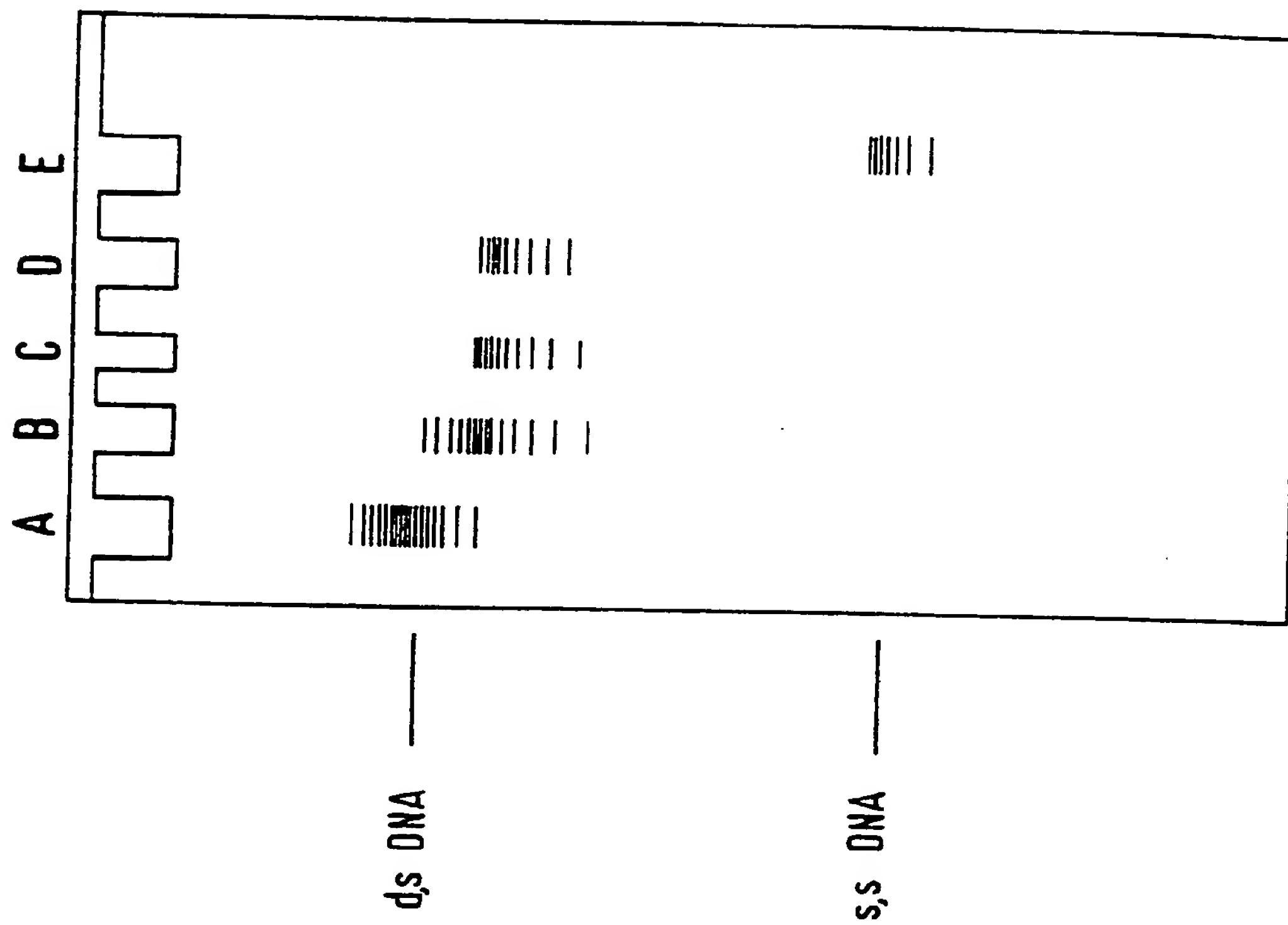
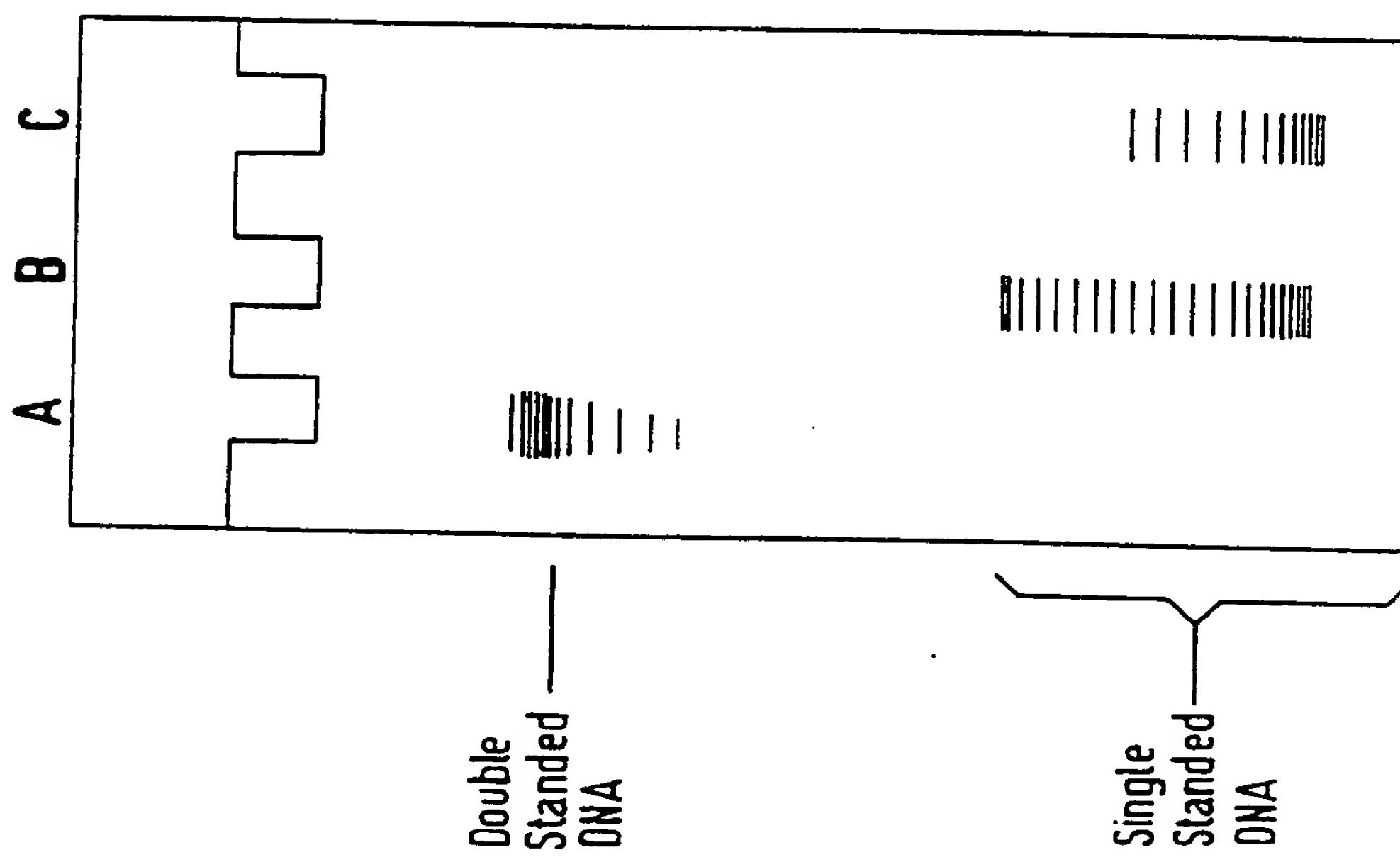


FIG. 2



3 / 6

FIG. 5

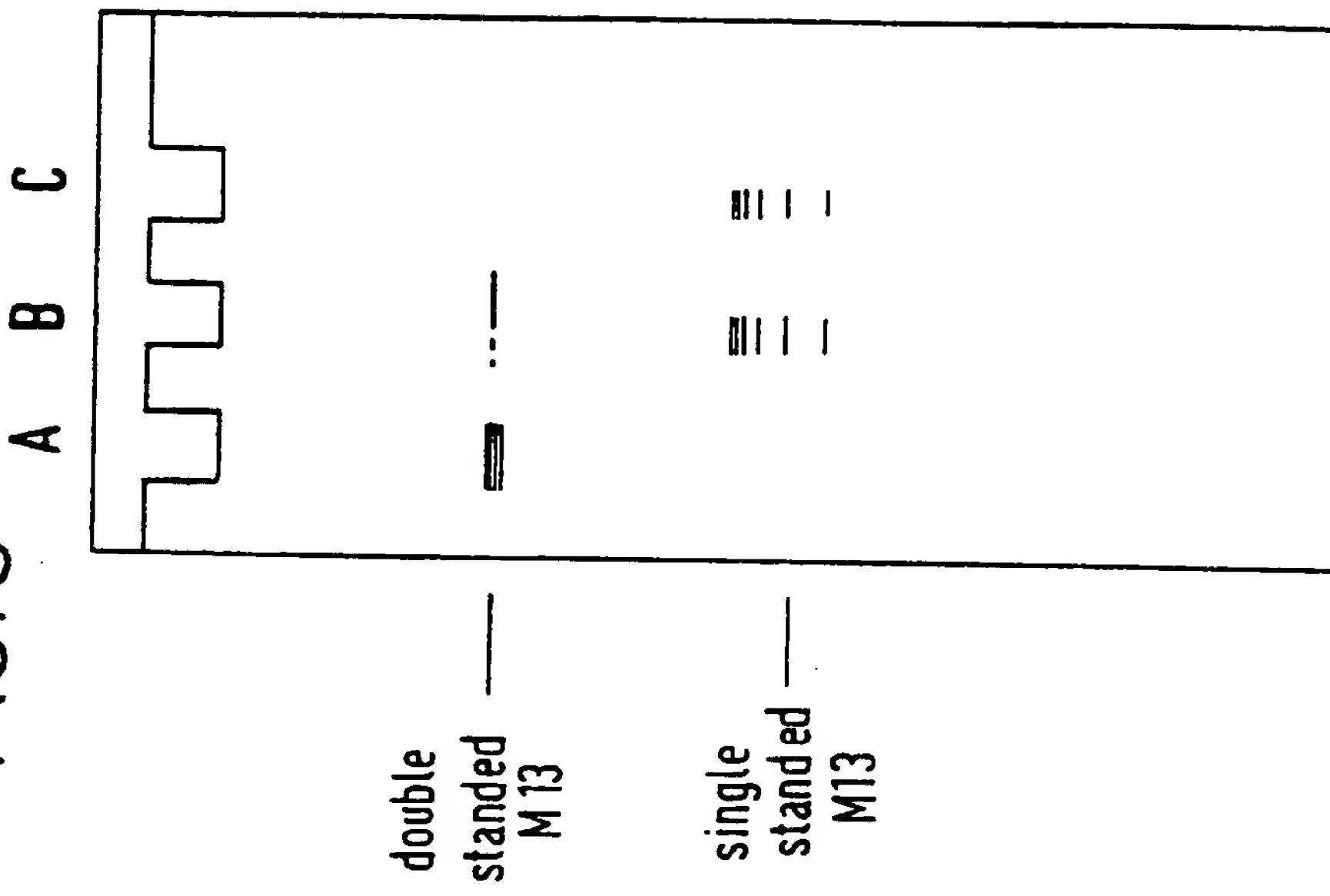


FIG. 4

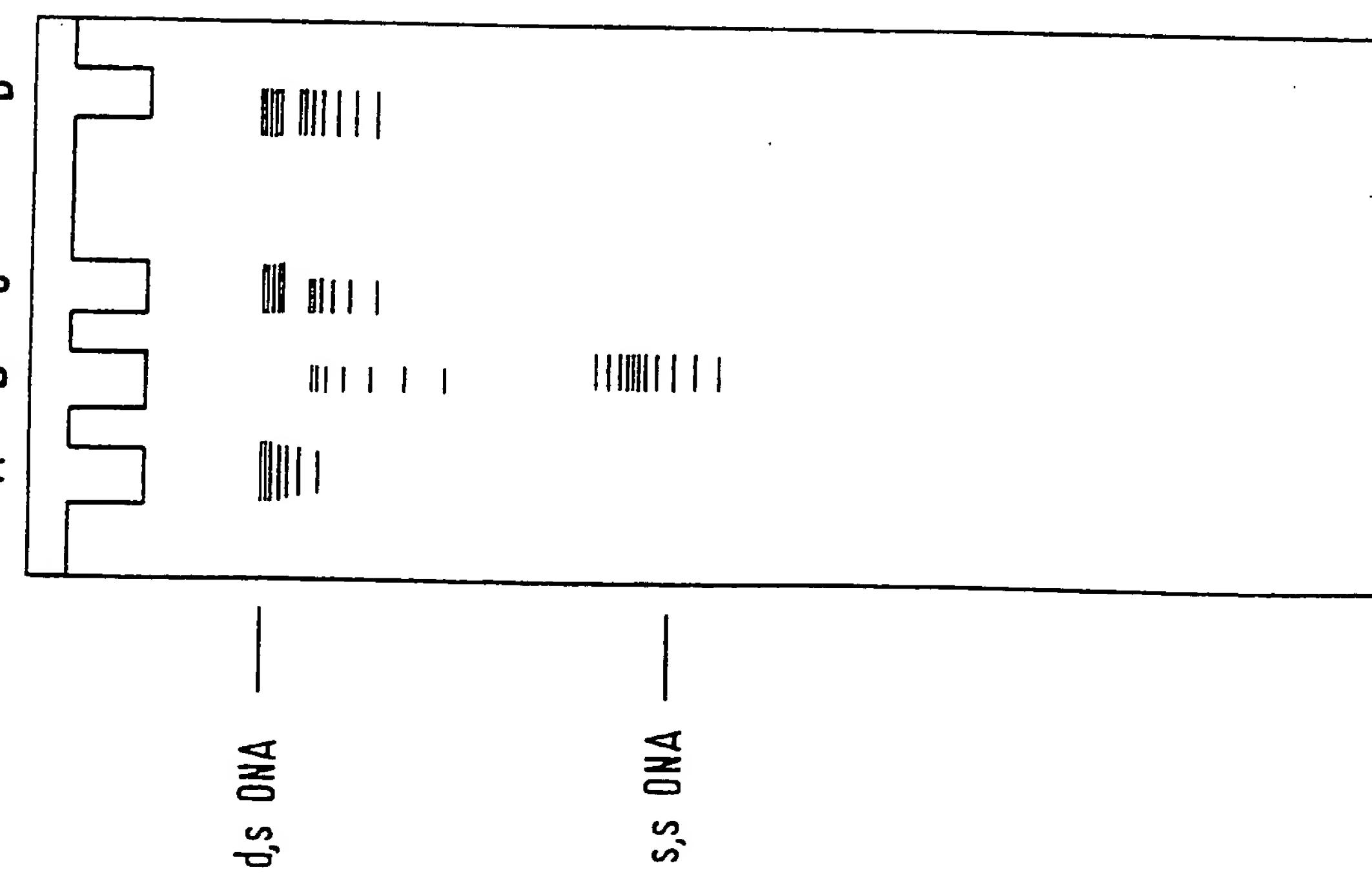
**SUBSTITUTE SHEET**

FIG. 6

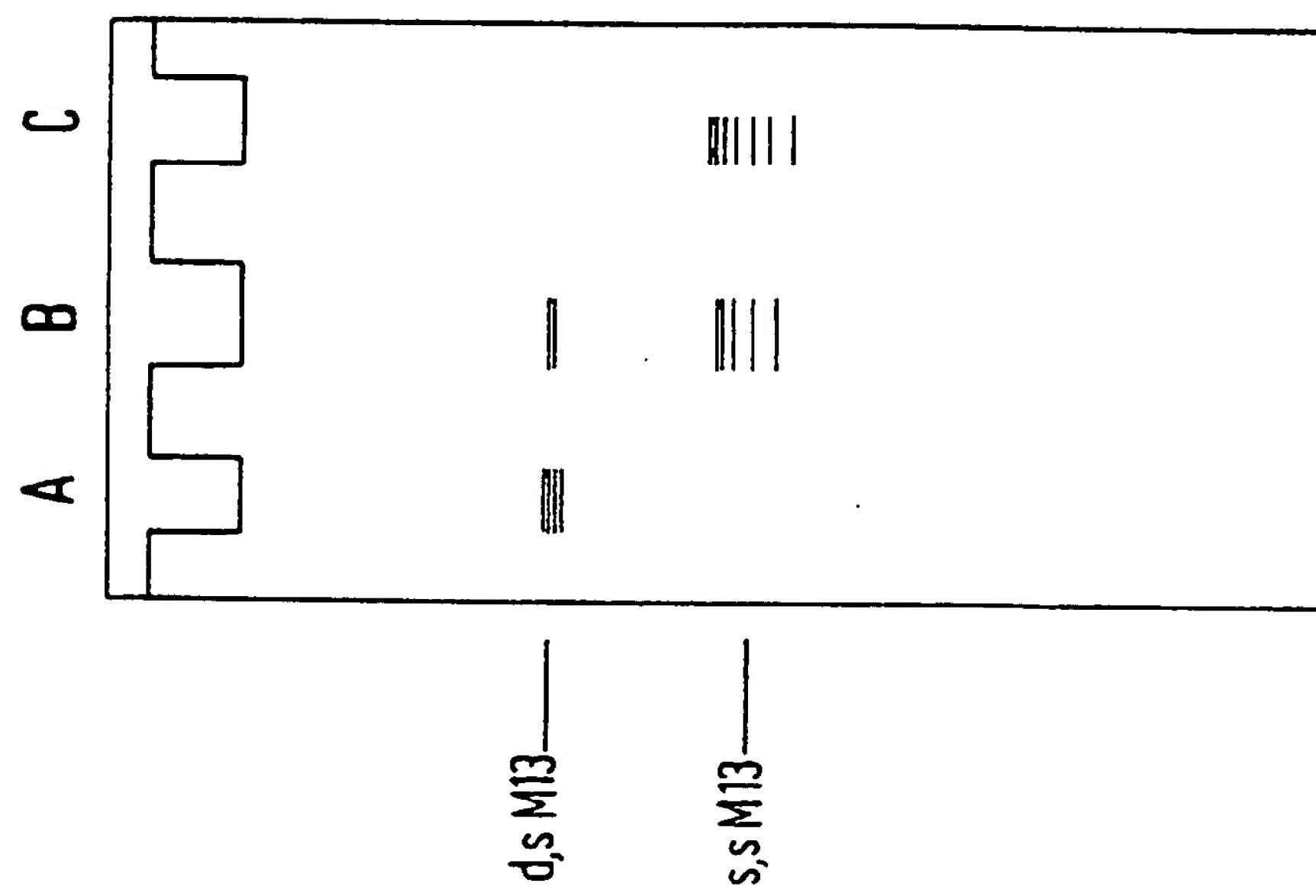
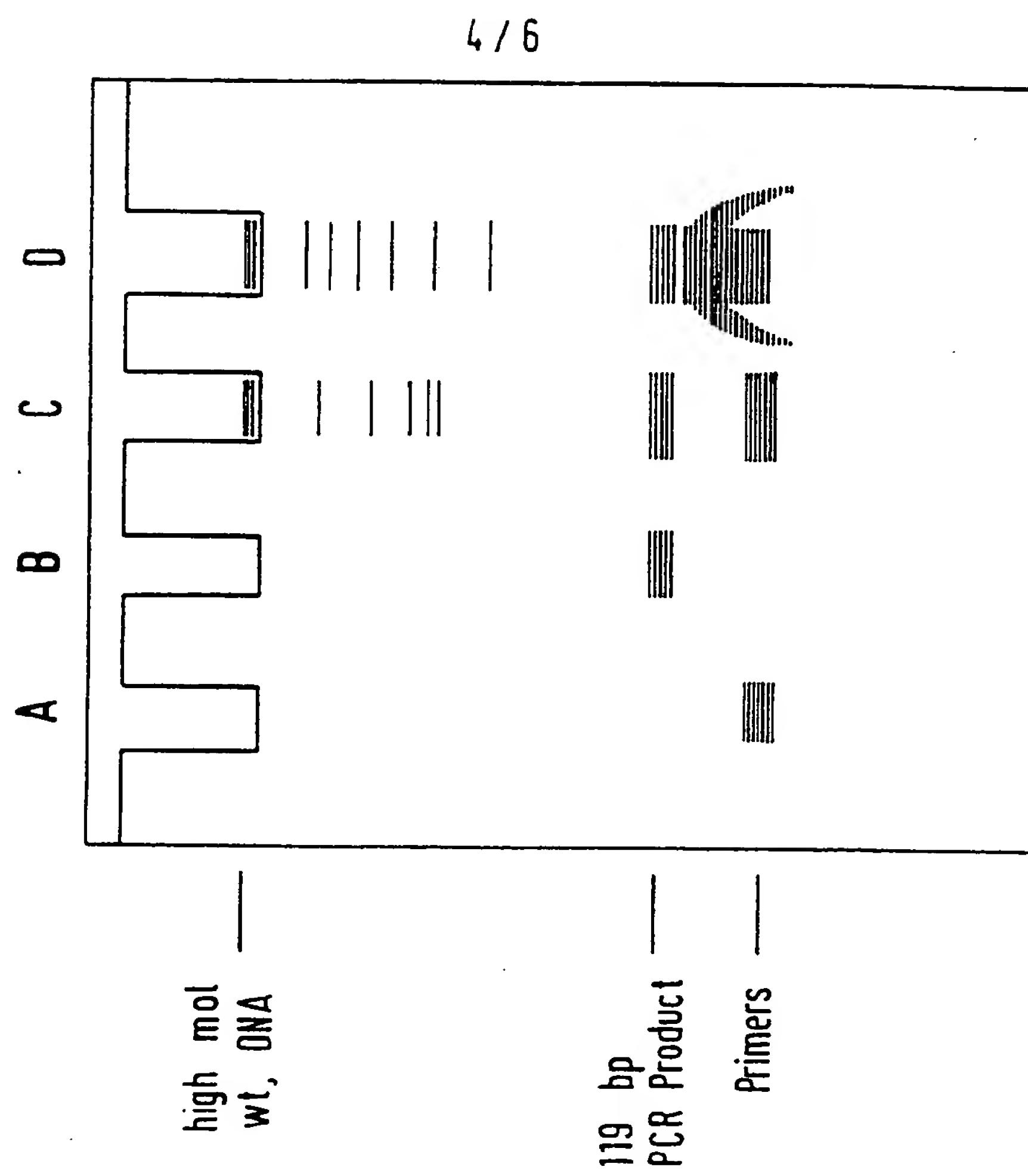
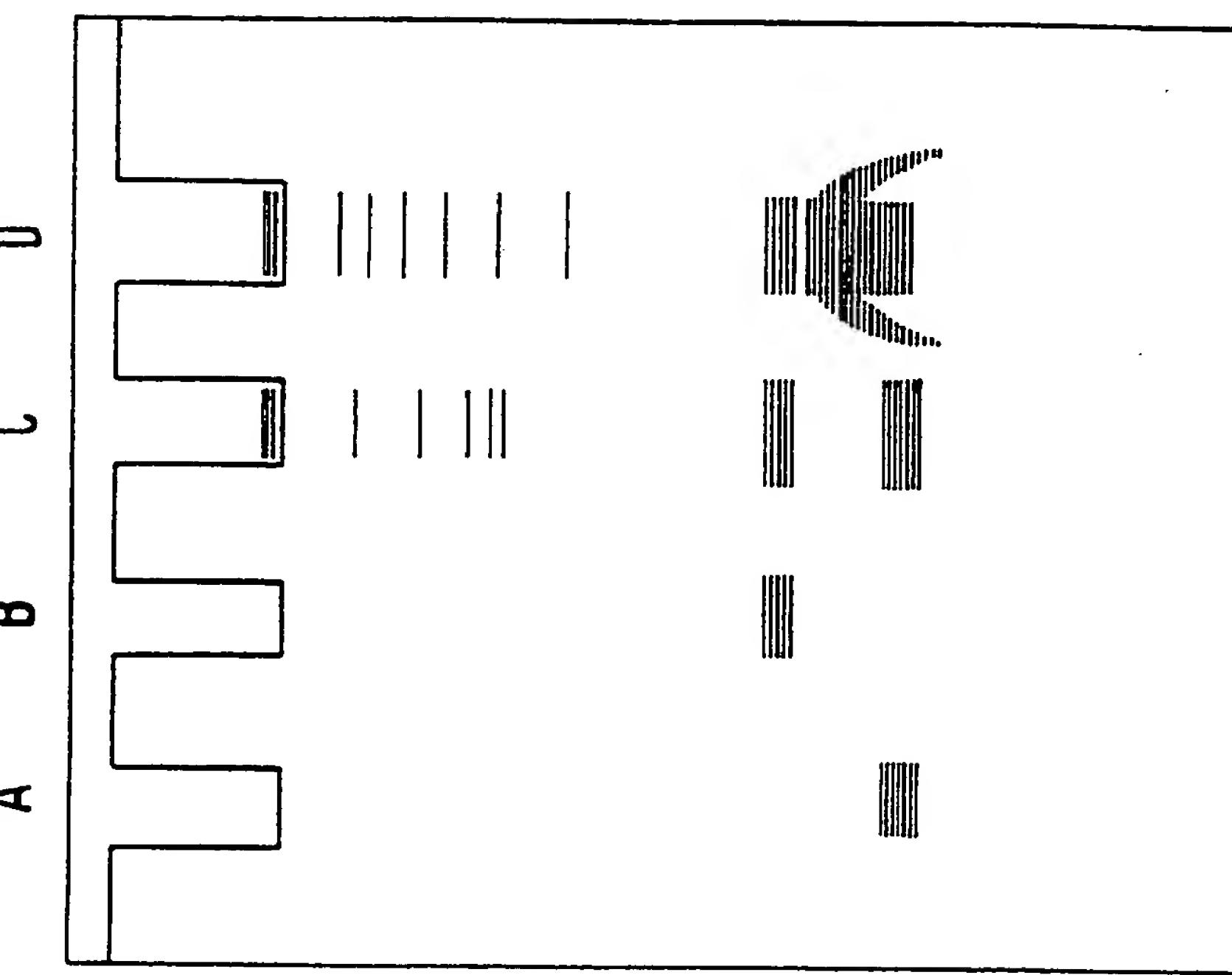


FIG. 7



4 / 6



5 / 6

FIG.8

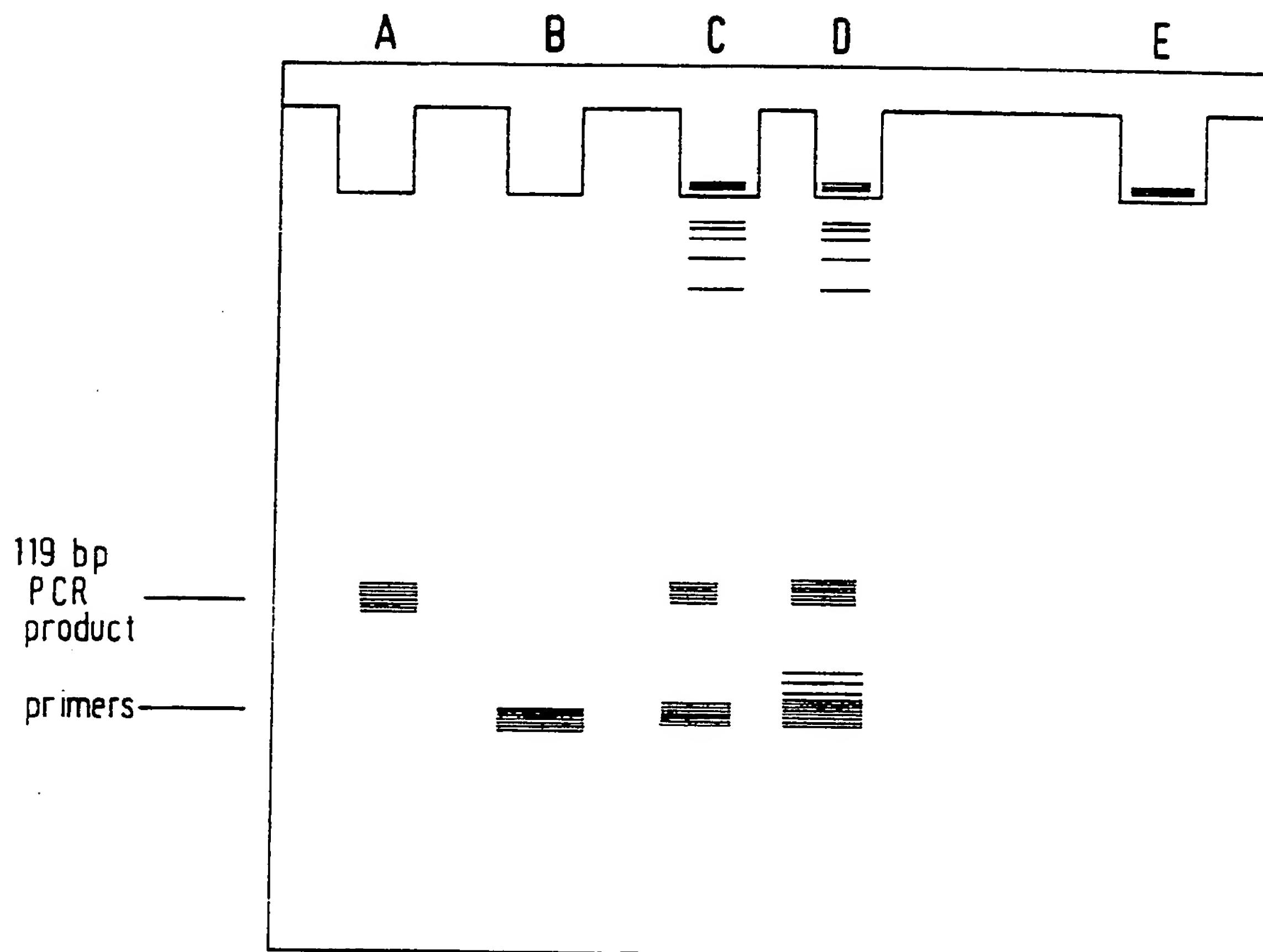


FIG. 9

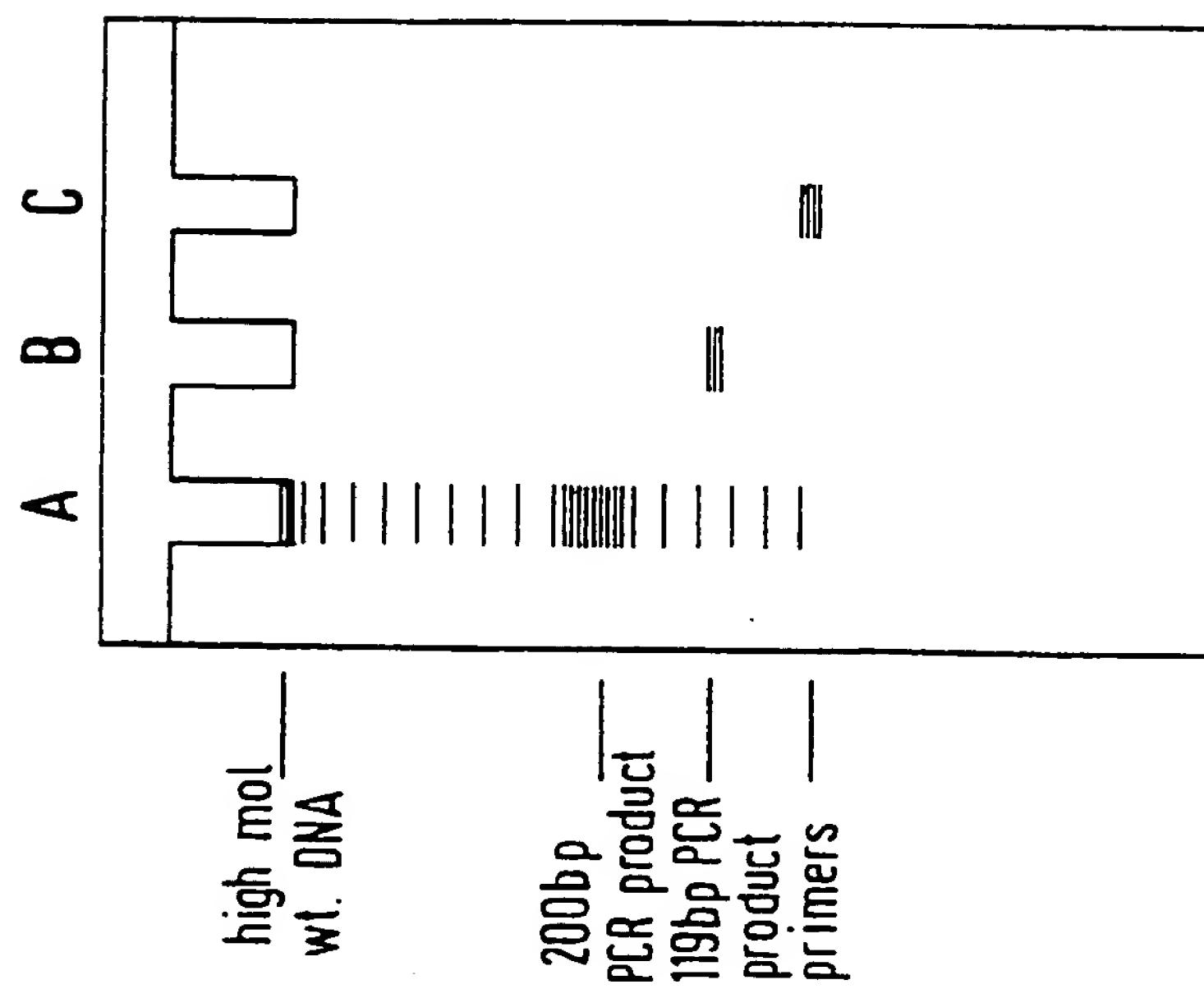
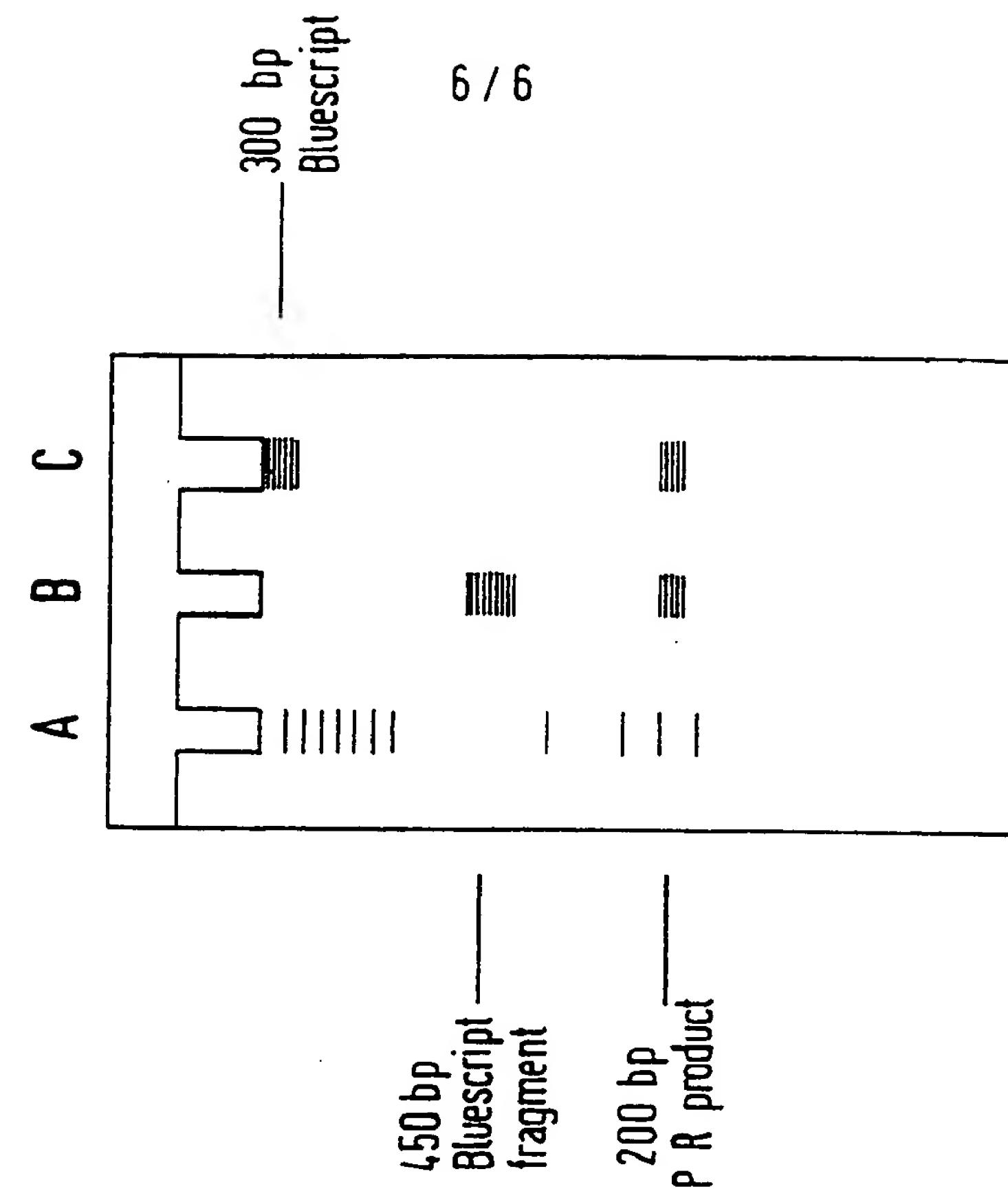


FIG. 10



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 91/01563

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 C12Q1/68; C12N15/10

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.C1. 5	C12Q ; C12N

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	BIOPHYSICAL CHEMISTRY vol. 4, no. 1, January 1976, AMSTERDAM pages 79 - 92; V. BRABEC ET AL.: 'Interaction of nucleic acids with electrically charged surfaces II. Conformational changes in double-helical polynucleotides' see abstract see page 80, left column, line 4 - line 13 see page 88, right column, line 4 - page 91, left column, line 14 ---- -----	1
A	----- -----	20,21

<sup>10</sup> Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

<sup>11</sup> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

<sup>12</sup> "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

<sup>13</sup> "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

<sup>14</sup> "&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

1

18 DECEMBER 1991

Date of Mailing of this International Search Report

36 JAHI 1992

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

LUZZATTO E.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF ELECTROANALYTICAL CHEMISTRY AND INTERFACIAL ELECTROCHEMISTRY vol. 88, no. 3, 25 April 1978, LAUSANNE pages 373 - 385; V. BRABEC ET AL.: 'Interactions of nucleic acids with electrically charged surfaces. Part IV. Local changes in the structure of DNA adsorbed on mercury electrode in the vicinity of zero charge' cited in the application see the whole document esp. abstract, page 383, line 30-page 385, line 15 ---	1
A	BIOELECTROCHEMISTRY AND BIOENERGETICS vol. 156, no. 2-3, November 1983, LAUSANNE pages 245 - 255; V. BRABEC: 'Conformational changes in DNA induced by its adsorption at negatively charged surfaces. The effect of base composition in DNA and the chemical nature of the adsorbent' see the whole document, esp. abstract and conclusions ---	1
A	BIOPHYSICS OF STRUCTURE AND MECHANISM vol. 1, no. 1, 1974, BERLIN pages 17 - 26; P. VALENTA ET AL.: 'The electrochemical behaviour of DNA at electrically charged interfaces' see page 23, line 26 - page 24, line 2 ---	

Failed Pages Report for Job 86413 Printed At 11:27:51 on 13/05/98

Series	Patent	Type	Page	Result
=====				
WO	09204470	A1	20	Page printed badly but recovered correctly